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The Study of the Formation of Oligomers and Amyloid Plaques from Amylin by Capillary Electrophoresis and Fluorescent
Microchip Electrophoresis

An Undergraduate Honors College Thesis

in the

Department of Biological Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

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The Study of the Formation of Oligomers and Amyloid Plaques
from Amylin by Capillary Electrophoresis and Fluorescent
Microchip Electrophoresis

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Abstract

Amylin, a pancreatic β -cell hormone, was the focus of this research project. This hormone is co-localized and co-secreted with insulin in response to nutrient stimuli. The hormone inhibits food intake, gastric emptying and glucagon secretion. Insulin and amylin appear to complement each other in the control of plasma glucose levels. Human amylin has a propensity to self-aggregate and to form insoluble bodies. In type 2 diabetes, amylin forms pancreatic islet amyloid plaques, which are found in 90% of people with the disease. It is still uncertain whether or not the deposition of amylin is a primary, or a secondary, event in the development of type 2 diabetes. Oligomers of amylin have been recently suggested as the cytotoxic cause of cell death; this is in opposition to larger amyloid deposits being the cause of β -cell deaths. This project examined the aggregation of Amylin. By using capillary electrophoresis and microchip electrophoresis we attempted to determine the earliest time points of amylin oligomer formation and monitor them as they continue to increase in size. The successful development of this method could allow researchers to better understand the cytotoxicity of amylin oligomers and in turn lead to better treatments and potentially earlier diagnosis for type 2 diabetes patients. Our current research was able to see Amylin aggregation with capillary electrophoresis, however the optimized conditions to best characterize the Amylin aggregates still needs to be determined. The microchip studies were not pursued far enough to observe Amylin aggregates through that process.

Introduction

Plasma glucose concentrations are determined by the rate of glucose leaving the bloodstream (Rd, or glucose disposal) and the rate of glucose entering the bloodstream (Ra, or glucose appearance).[1] Glucose production by the liver and the glucose taken in from meals accounts for the Ra in the bloodstream. Insulin, secreted by pancreatic β -cells, primarily controls the Rd (glucose disappearance) functions. Glucagon is secreted by pancreatic α -cells in response to stress and hypoglycemia. This hormone increases Ra by forcing the liver to secrete glucose and stimulating gluconeogenesis.[1] Amylin, a second β -cell hormone, is co-localized and co-secreted with Insulin in response to nutrient stimuli.[1] Amylin functions in ways that control Ra. The hormone inhibits food intake, gastric emptying and glucagon secretion. This suggests that Insulin, which primarily controls Rd, and Amylin, which primarily controls Ra, appear to complement each other in the control of plasma glucose levels.

Human Amylin has a propensity to self-aggregate and to form insoluble bodies. In type 2 diabetes, Amylin forms pancreatic islet amyloid plaques, which are found in 90% of people with the disease.[1] In an animal model of diabetes, the causal role of Amylin in the development of type two diabetes has been strongly supported.[2] It is still uncertain whether or not the deposition of Amylin is a primary, or a secondary, event in the development of type 2 diabetes. Oligomers of Amylin have been recently suggested as the cytotoxic cause of cell death; this is in opposition to larger amyloid deposits being the cause of β -cell deaths.[2]

Amylin Cytotoxicity

Mirzabekov, et al. studied the role of Amylin in the formation of voltage-dependent ion-permeable channels in the phospholipid bilayer.[3] This channel formation is dependent upon lipid membrane composition, ionic strength and membrane potential. At 1-10 μM , cytotoxic human Amylin dramatically increases the conductance of lipid bilayer membranes. This suggests that channel formation is the mechanism of cytotoxicity for Amylin. The researchers also tested Rat Amylin, which is highly homologous to human Amylin, and showed no formation of pores. Therefore, the formation of these pores is not caused by length or general sequence of the polypeptide. The researchers summarize several reasons that explain why this channel formation contributes to cytotoxicity. First, channel formation occurs at concentrations comparable with those required for cytotoxicity. This shows a possible correlation between the two events. Second, the Amylin channels are poorly selective channels. They let through many ions that normal membrane channels do not. This can lead to disruption of ionic homeostasis. This includes influxes of Ca^{+2} and Na^{+} and effluxes of K^{+} . Prolonged elevations of Ca^{+2} can potentially lead to cellular damage and even cell death. In addition to the potentially serious effects of these ionic changes, the cell would face increased energy demands as various pumps and exchangers attempted to compensate for these

ionic disturbances. Amylin not only kills Beta cells but also is able to kill other cell types. This non-specificity matches with a pore formation model of toxicity. The amount of amyloid found in pancreatic islets is proportional to the amount of Beta cell destruction and to the Insulin requirements of the patient. Thus increased Amylin deposition may lead to increased channel formation and cell destruction, thereby increasing the Insulin requirement of the patient.

Amylin Inhibition Studies

A promising area of research for the understanding and treatment of Amylin related disease is inhibitor studies. Various inhibitors have been studied and shown to either prevent larger species from forming or shown to make the Amylin aggregates non-toxic to the cells.

Insulin is a kinetic but not a thermodynamic inhibitor of Amylin aggregation

Previous studies have suggested that Insulin inhibits Amylin aggregation *in vitro*. [4] Contradictory to this, other studies have shown that Insulin could promote amyloid formation and enhance the binding of Amylin to preformed fibrils. [5] This shows that the effects of Insulin on Amylin could be multi-dimensional. Cui, et al. [6] found that both the above results do happen *in vitro* using light scattering. In the short-term, Insulin does inhibit Amylin aggregation. As the time period lengthens, Insulin actually promotes Amylin fibrillization. These effects were enhanced by a greater Insulin/Amylin ratio. The researchers found that the promotional effects were a result of copolymerization of the two proteins. The researchers also found that Insulin significantly enhanced fibril formation by interacting with Amylin monomers or oligomers rather than existing Amylin fibrils.

These results show that the previous hypothesis of Insulin inhibition as a reason for the normal function of Amylin in healthy adults is not a completely valid hypothesis. The research shows that the interaction between Amylin and Insulin is a complex one. While a study of short-term effects could show certain positive results from Insulin interaction a long-term study of the same type can show opposite results. With inhibitors it is important to fully understand their mechanisms before making hypothesis about their functions in nature.

Beta-Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red

Lorenzo, et al. studied the effect of Beta-Amyloid on rat hippocampal cultures. [7] They also studied the effect of Amylin on rat pancreatic cells. While studying the affects on the cells the group also tested how Congo red affected the growth of the fibrils. The group demonstrated that the addition of Amylin to the cells resulted in marked degenerative changes with $62 \pm 3\%$ islet cell death after 24 hrs. The addition of Congo Red was shown to completely inhibit Amylin toxicity. The cells were centrifuged after the study and were shown to have Amylin peptide that had converted entirely to the

insoluble form of Amylin fibrils. This demonstrates that the dye did not inhibit Amylin fibril formation. Congo Red did however render the Amylin non-toxic. The results suggest Congo Red is a general inhibitor of amyloid fibril toxicity. For Beta-Amyloid, the experiments showed that the neurotoxicity is mediated by the amyloid fibril. In the cell experiments, there was a marked difference between immature diffuse plaques and compact mature plaques. The immature plaques are not associated with neurodegenerative changes and are Congo Red negative. The mature plaques are associated with neurodegenerative changes and stain Congo Red positive. The group claims that the cyto-protective effect of Congo Red for Beta-Amyloid neurotoxicity and Amylin islet cell toxicity suggest that different amyloid fibrils have similar toxic mechanisms. With these results it is possible therapeutic agents that inhibit one certain type of amyloid fibril formation may have therapeutic effects for several types of amyloidosis.

These results show a greater confidence in amyloidosis as the cause of pancreatic cell death. They also show a type of dye that binds to fibrils and not just stains the fibrils but inhibits further growth.

Interaction of the anthracycline 4'-iodo-4'-deoxydocorubicin (IDOX) with amyloid fibrils: Inhibition of amyloidogenesis

All types of amyloidosis are structurally characterized by the cross Beta-pleated sheet conformation of the fibrils, irrespective of their biochemical composition.[8-9] The research of Merlini, et al. was sparked off by the clinical observation that IDOX could induce amyloid resorption in patients with immunoglobulin light chain amyloidosis.[10] The group found that IDOX binds strongly to all of the fibrils tested. They did not test Amylin fibrils. They did test Beta-Amyloid. The group hypothesized that IDOX exerts its beneficial effects through the inhibition of fibril growth. The affinity of IDOX for amyloid is reminiscent of that of Congo red but they do not bind competitively when both applied. The IDOX binds to amyloid fibrils specifically and with high affinity. The binding both in vitro and in vivo affects the fibrils by inhibiting further growth and by promoting resorption. The obvious drawbacks of the treatment with IDOX are its toxicity. Nontoxic analogs could possibly be created that could be used as a therapeutic approach and a potential diagnostic tool for multiple types of amyloidosis.

This drug has not been tested on Amylin and seems to have an effect on all types of Amyloid fibrils. The drug seems to be a good choice to study on Amylin. The issue with this fibril inhibitor is the same as Congo red in that it toxic but the paper does suggest that it is possible to make nontoxic analogs.

A Two-Site Mechanism for the Inhibition of IAPP Amyloidogenesis by Zinc

The dysregulation of metal ions in amyloid- related diseases has received more attention after the discovery of a high prevalence of metal ions in amyloid deposits. It has been shown that amyloidogenic proteins, such as amyloid beta (A β), α -synuclein, and

mammalian prion protein, have transition metal binding sites that affect aggregation rates.[9] However, the interactions of metals with different proteins do not produce the same outcomes. For example, while copper has been shown to promote the formation of A β fibers, copper inhibits the formation of IAPP fibers. The role of zinc in type II diabetes and hIAPP aggregation is less understood, as both zinc supplements and zinc chelation have been shown to decrease β -cell death and symptoms of type II diabetes. The research of Salamekh, et al. suggests that zinc has a variety of effects on β -cells and stress the need for further investigation.[11] Zinc has already been shown to affect a variety of processes involved in glucose homeostasis, including storage and excretion of Insulin, glucose-stimulated secretion cascades, and paracrine communication with α -cells; however, little is known about zinc's effect on IAPP-induced β -cell death.

The researchers conducted various studies of zinc's effects on Amylin. The group showed that zinc has an affinity for Amylin monomers. The affinity is much less for amyloid fibrils. Zinc is displaced upon fibril formation. The research group found the existence of two zinc-binding sites, one high and one low affinity. This correlates with the kinetic data that show that zinc inhibits fiber formation at low concentrations, but the degree of inhibition significantly decreases at millimolar concentrations. After saturation of the high-affinity, inhibitory binding site, the second low-affinity binding site becomes occupied, which accelerates fiber formation.

This study shows some of the effects of zinc on Amylin. They are complex effects such as the ones observed in Amylin's interaction with Insulin. The way that zinc affects Amylin *in-vitro* is harder to comprehend. Amylin can experience anywhere between millimolar and picomolar concentrations of zinc during its existence.

This Study

As previous research shows, amylin aggregates appear to be cytotoxic and have been implicated in the development of type 2 diabetes. Therefore, a method is needed that can detect the early formation of Amylin oligomers and monitor their progression. Capillary and microchip electrophoresis are promising methods of detection, but need to be able to detect Amylin at physiological levels.[12,13] Since fluorescent dyes can be sensitive at picomolar concentrations, we plan to use a FAM label to study Amylin aggregation.

Experimental Aims

Initial studies with capillary and microchip electrophoresis were conducted. Various preparation methods and run settings were explored to observe Amylin growth. Initially, it was also planned to use capillary or microchip electrophoresis to evaluate various inhibitors, such as the ones described in the background, to observe what size aggregates are inhibited by what inhibitors. However, due to unanticipated complications, these experiments were not able to be completed.

Materials and Methods

Aliquot Preparation for Amylin

Dried Amylin was obtained from Anaspec (#60804 Amylin (1-37), human) in 1 mg vials. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was added to the vial of Amylin such that the concentration was 1 mM (256 μ L HFIP to 1 mg Amylin). The vial was left with HFIP at room temperature for 60 min. The vial was separated into individual aliquots such that the contents of each micro-centrifuge tube will be 0.0271 mg/tube (6.94 μ L). The tubes were evaporated overnight and then stored in a -80°C freezer.

The preparation procedure was the same for FAM labeled Amylin. Dried FAM labeled Amylin was obtained from Anaspec (5-FAM-Amylin (1 - 37), human #64448-05) in 0.1 mg vials. It was prepared in a 0.1 mM concentration (235 μ L HFIP to 1 mg Amylin). Each tube was made to contain 0.006775 mg/tube (15.92 μ L).

Capillary and Microchip 30 μ M Sample Preparation

One aliquot of 0.0271 mg Amylin and one aliquot of 0.00675 mg FAM-Amylin were used. 11.56 μ L of 5 mM NaOH was added to the Amylin vial and 3.15 μ L was added to the FAM-Amylin vial. The NaOH was dissolved for 30 min. The solution was diluted into 40 mM Tris-HCl (pH 9.0). 196.56 μ L was added to the Amylin vial and was added to the 53.62 μ L FAM-Amylin vial. The solution was then supplemented with 10 mM NaCl. 23.13 μ L of 100mM NaCl was added to the Amylin vial and was added to the 6.31 μ L to the FAM-Amylin vial. The vials are then combined to get the desired ratio. For 50%, 173.44 μ L of Amylin was combined with 47.32 μ L of FAM-Amylin. For 20%, 48.56 μ L of Amylin was combined with 52.99 μ L of FAM-Amylin.

Capillary Array Coating Procedure

For each step, the desired solution was loaded into syringes and pushed through the syringe pump (KD Scientific #780220V). The solution was pushed through at a very slow rate for the desired time. After all solutions had been used, the array was to stored in 40 mM Tris-HCl pH 9

De-ionized water was run for 15 minutes. Then 1 M HCl was run for 15 minutes. Then 0.1% PHEA (dissolved in de-ionized water) was run for 20 minutes. Finally, de-ionized water was run for 15 minutes.

Capillary Electrophoresis Settings

The machine used was an Applied Biosystems 3130 Genetic Analyzer #627-0040. The buffer used was 40 mM Tris-HCl pH 9. The separation polymer was 0.5% PHEA (dissolved in 40 mM HCl pH 9). The injection voltage is 7 kV with an injection time of 10 seconds. The run voltage was 7 kV with a run time of 9000 seconds.

Microchip Coating Procedure

For each step, the given solution was pulled through microchip channels with a vacuum pump. When the time had elapsed, the vacuum pump was used to pull out the solution. After all solutions had been used, the chip was stored dry.

De-ionized water was run for 15 minutes. Then 1 M HCl was run for 15 minutes. Then 0.1% PHEA (dissolved in de-ionized water) was run for 20 minutes. Finally, de-ionized water was run for 15 minutes.

Microchip Settings

The buffer used is 40 mM Tris-HCl pH 9. The separation polymer is 1% PHEA (dissolved in 40 mM HCl pH 9).

Run 1:

The injection voltage was 500 V for 20 seconds.

The separation voltage was 3000 V with a pullback voltage of 200 V.

Run 2:

The injection voltage was 500 V for 20 seconds.

The separation voltage was 3000 V with a pullback voltage of 100 V.

Run 2:

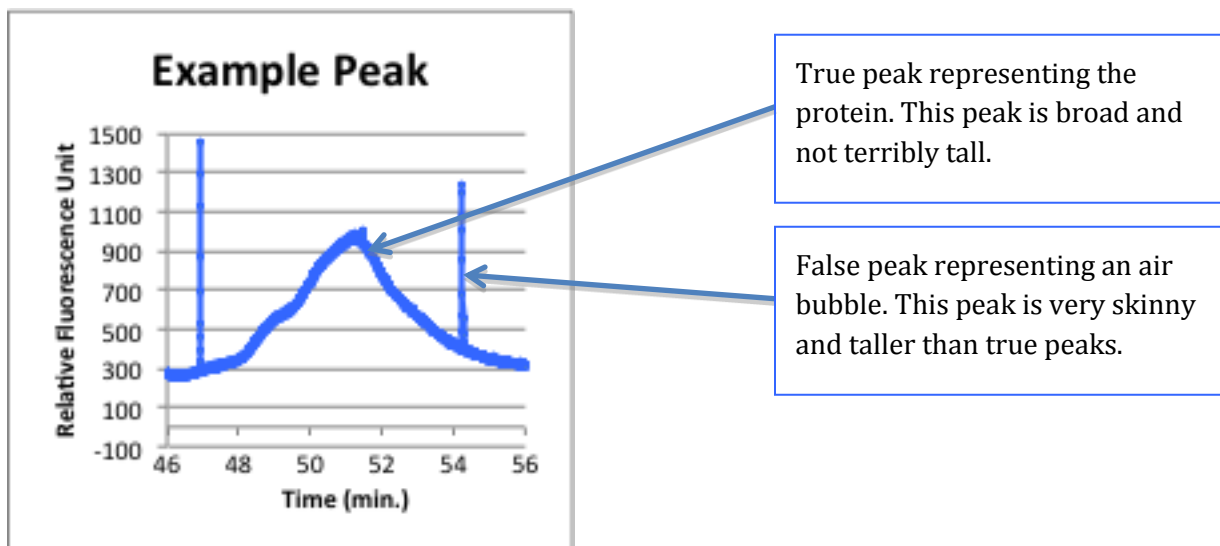
The injection voltage was 600 V for 20 seconds.

The separation voltage was 4000 V (-1000 V at one end 3000 V at other end) with a pull-back voltage of 100 V.

Results/Discussion

Capillary electrophoresis runs were conducted at different FAM-Amylin/Amylin ratios. The data from these runs were plotted with the run time versus the relative fluorescence unit at that time-point. The capillary electrophoresis apparatus works by creating an electrical gradient along the capillary. This gradient pulls the sample from where it is injected to a window in the capillary where a laser is shown onto the capillary. As the sample moves through the window, the FAM label absorbs the laser light and then fluoresces it back. The machine observes this as the relative fluorescence unit. When the graphs are plotted the peaks at later time points are species that have taken longer to pass through the capillary. With a single sample type (isoelectrically speaking), the only difference that would separate the peaks would be the size of the species. With the Amylin samples, earlier peaks should be samples of smaller size such as monomers and smaller sized oligomers. The later peaks should be larger size oligomers.

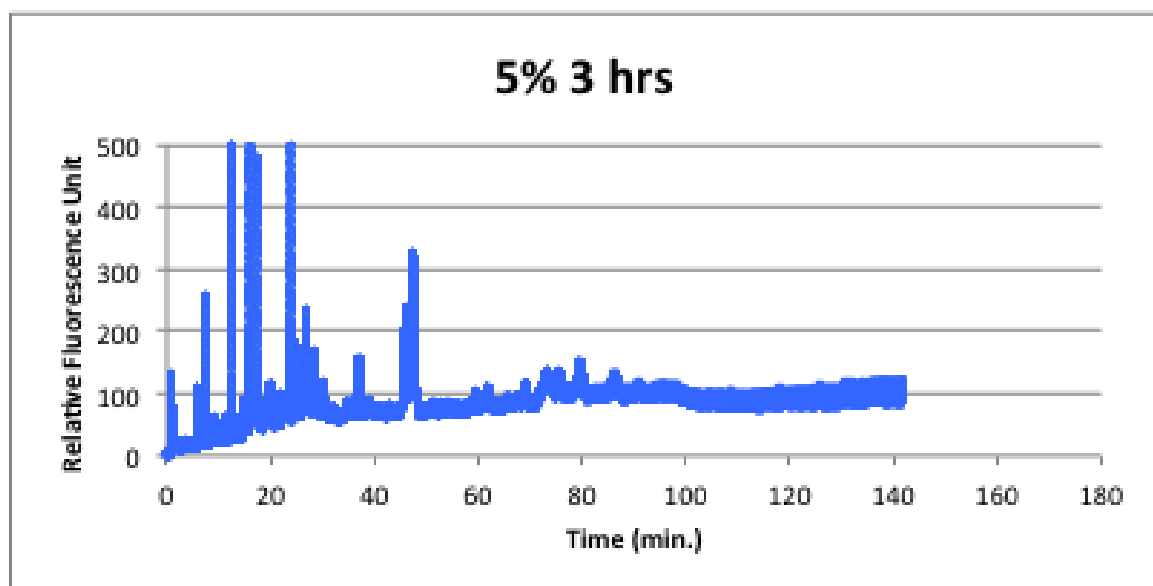
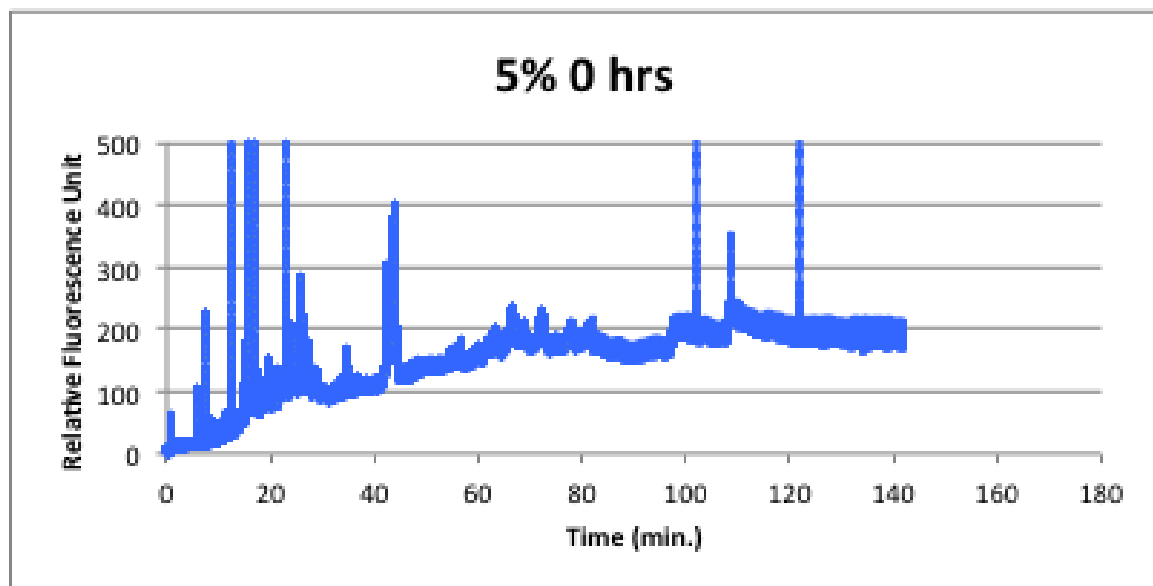
The following figure is an example of how true protein peaks are distinguished from noise and from bubbles. This is an important distinction to recognize since there is a large amount of this type noise in the graphs, especially the early times during the run.

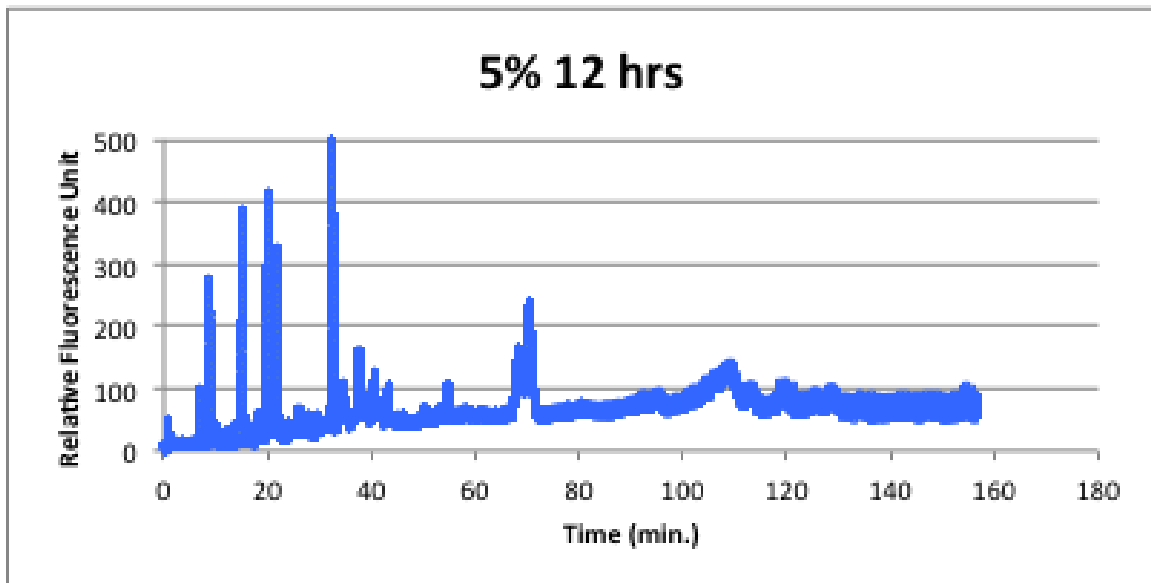
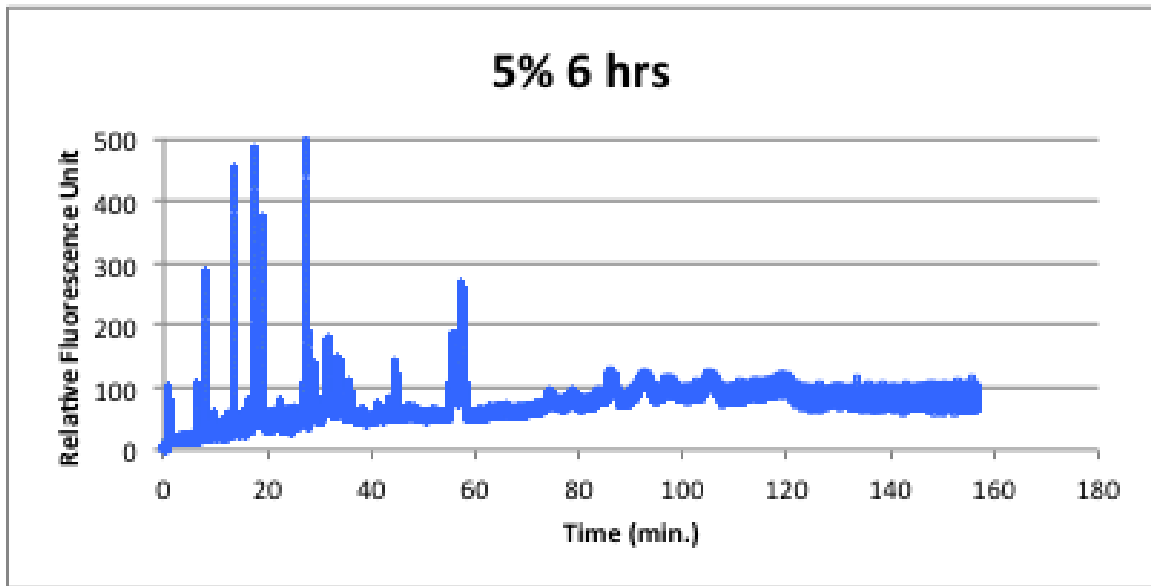


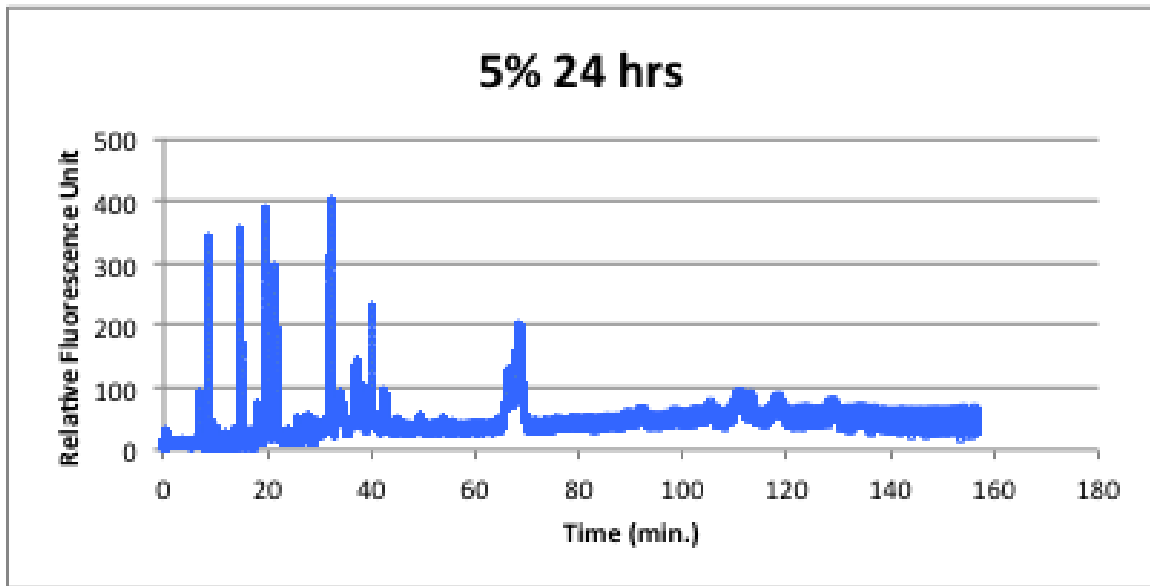
When the run conditions were first set up, conditions were used that had been refined for another protein. This preparation used 40 mM Tris HCl pH 8 as the buffer. Initial runs showed no peaks; suggesting that Amylin did not travel through the capillary. This inability to travel through the capillary was found out to be an issue of Amylin's pI. Amylin has a pI of 8.59. At pH 8, Amylin is actually positively charged and will not flow along the gradient of the electrophoresis machine. This means that Amylin's charge at this pH actually makes it want to migrate toward the cathode (negatively charged end that is located at the sample injection end of the capillary). We decided to raise the pH of the buffer to pH 9 making Amylin have a negative charge. This will cause Amylin to flow down the gradient towards the anode (positively charged end located on the end of the capillary that contains the detection window) and be detected by the instrument. When the buffer was changed, we forgot to change the PHEA polymer solution. So some of the following runs were done with a buffer of pH 9 and a polymer dissolved in a buffer of pH 8. This would make the sample experience a pH somewhere between pH 8-9, most likely closer to pH 9. At this pH, the Amylin should be very slightly negatively charged or not charged at all.

5% FAM-labeled Amylin in pH 8 Polymer

Run conducted using a ratio of 5% FAM labeled Amylin to 95% unlabeled Amylin.



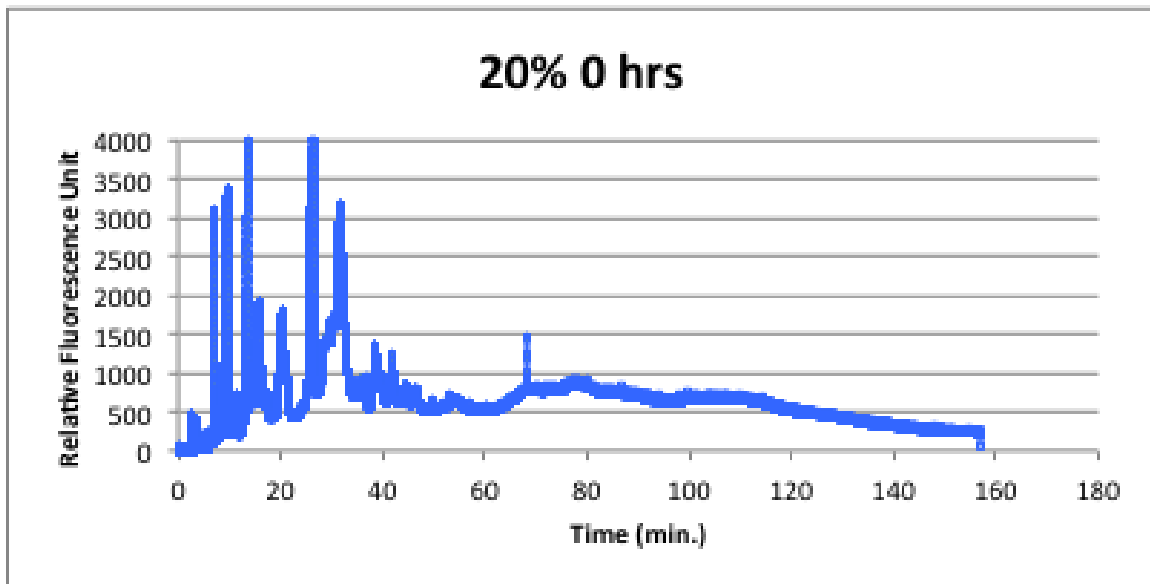


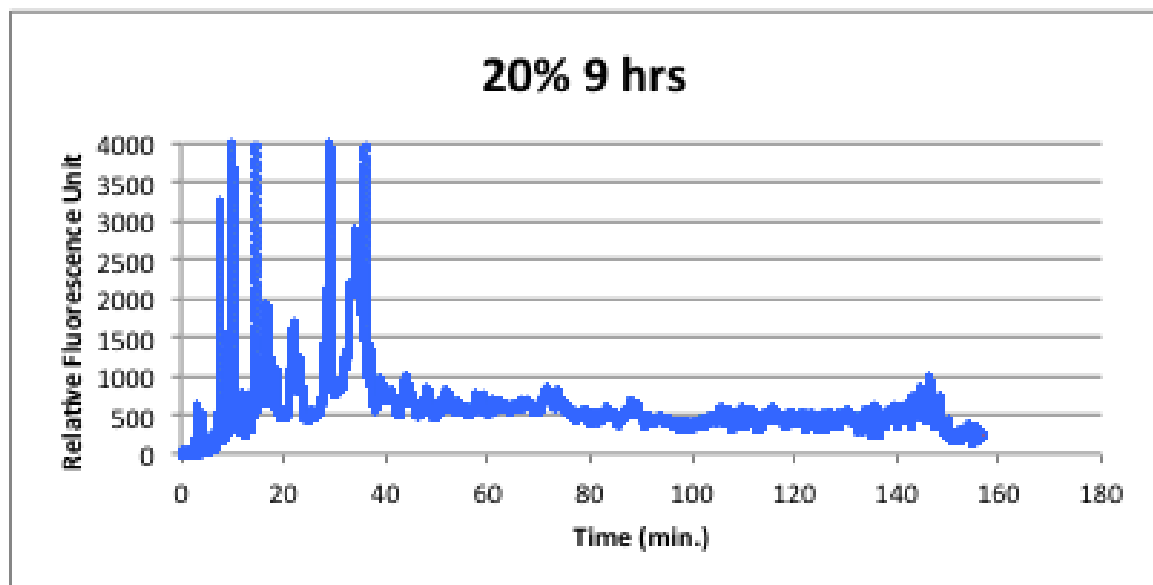
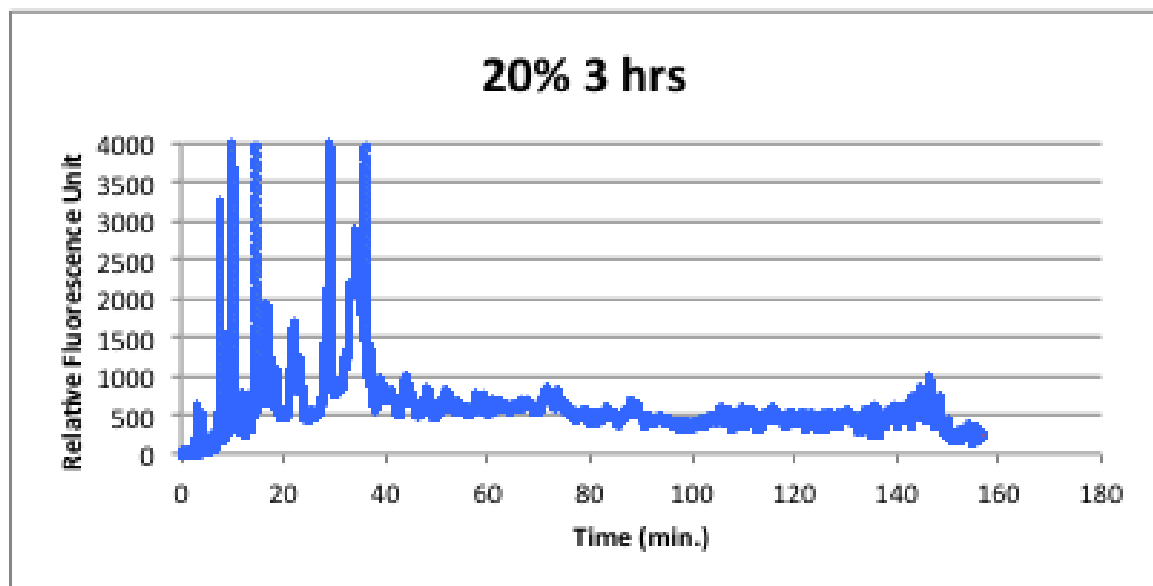


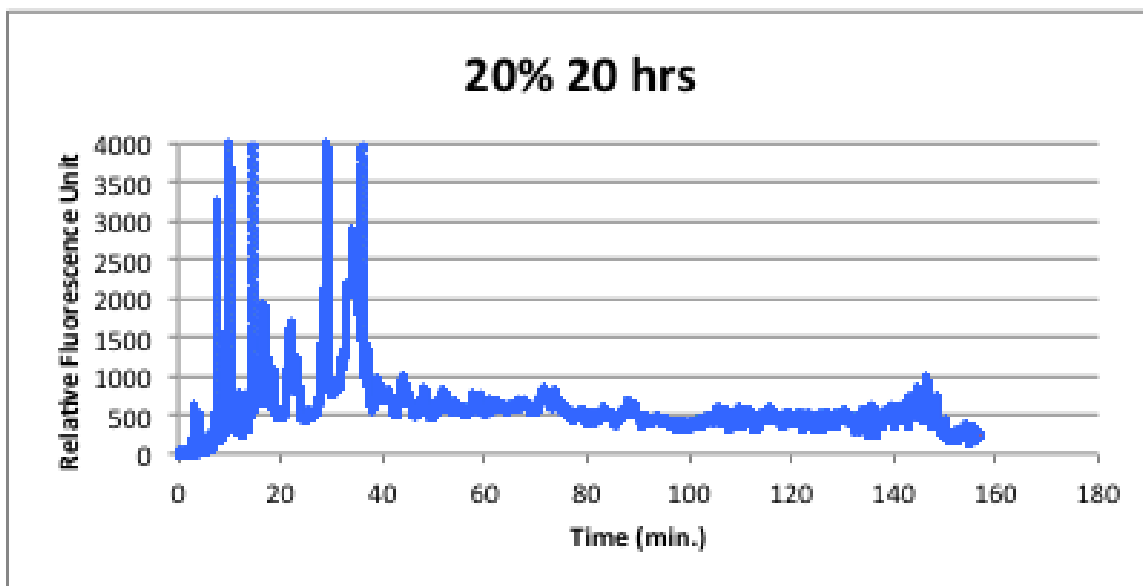
These test at 5% showed a low detection of the Amylin species. Multiple sharp peaks were detected which were most likely bubbles. Only a few peaks could be determined to be true Amylin peaks. The peak that elutes between 40-80 minutes in each graph can be observed as eluting at a later time point as the aggregation time increases from 0-24 hrs. This is an indication that the species in that peak is increasing in size as time increases.

20% FAM-labeled Amylin in pH 9 Polymer

Run conducted using a ratio of 20% FAM labeled Amylin to 80% unlabeled Amylin.



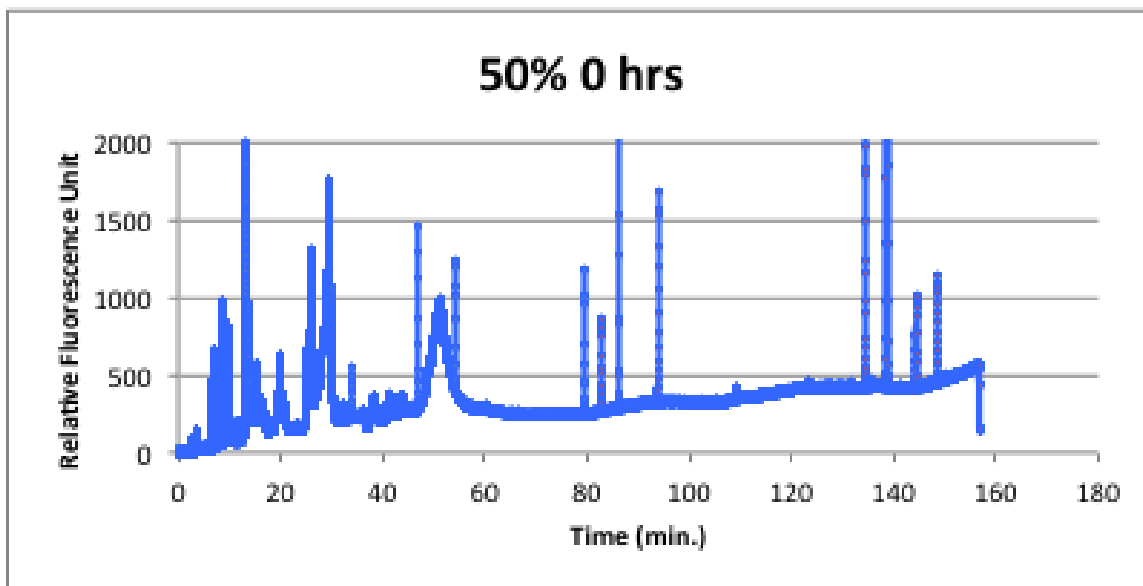




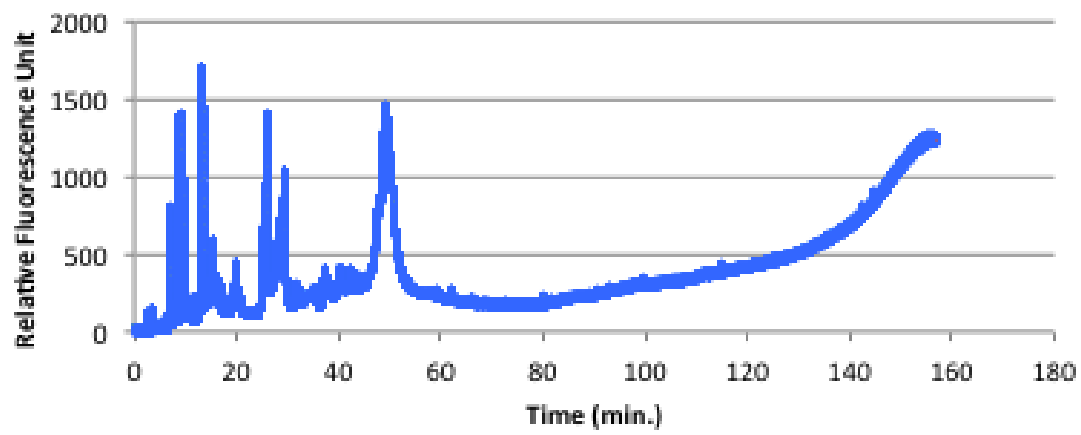
The runs at 20 % show multiple size species in each graph. Multiple broad peaks can be observed from 10-40 minutes. No change in peak elution times can be observed from 0-20 hrs.

50% FAM-labeled Amylin in pH 9 Polymer

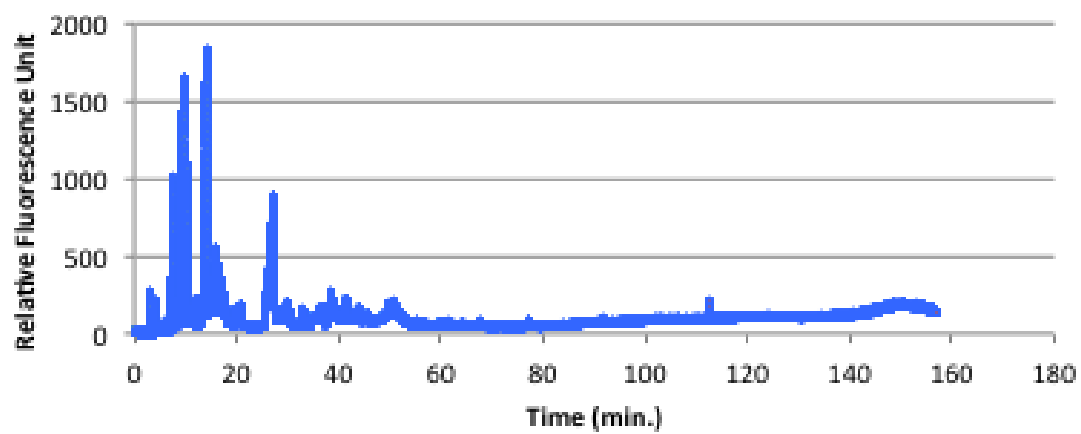
Run conducted using a ratio of 50% FAM labeled Amylin to 50% unlabeled Amylin.

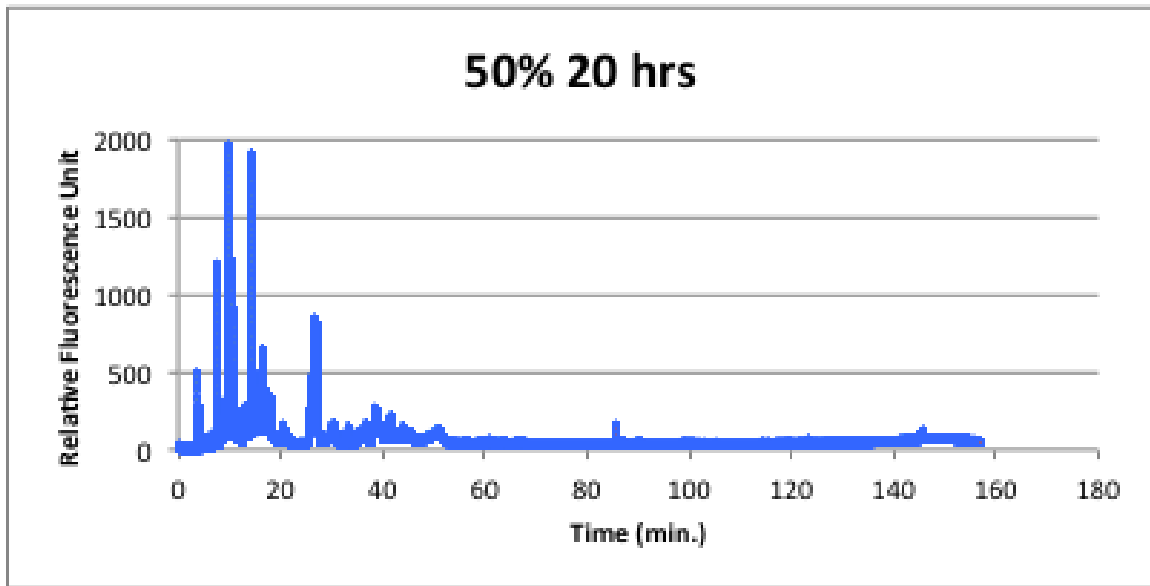


50% 3 hrs



50% 9 hrs

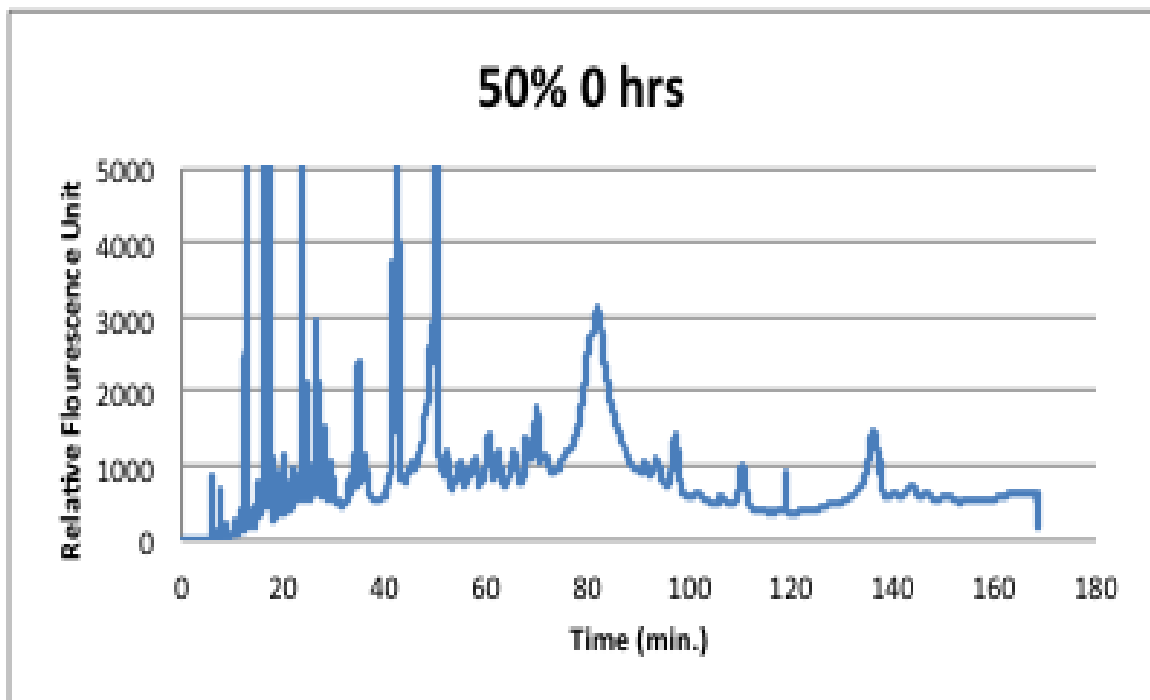




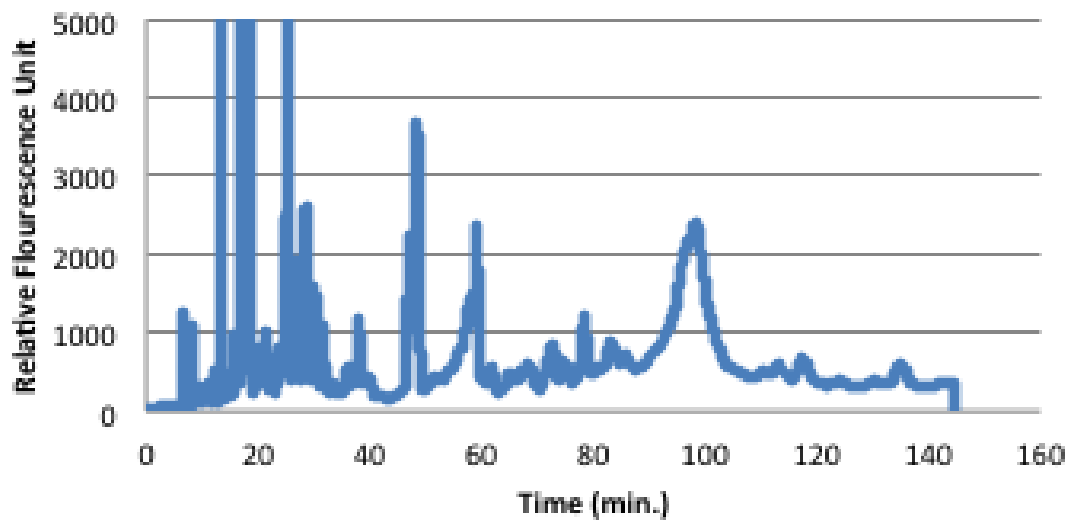
This 50% study shows multiple large peaks at 0 and 3 hrs. These peaks do not change elution time at the 9 and 20 hrs. The peaks do decrease in area. This could suggest that some of the species observed at the 0 and 3 hr time points have formed larger species that elute at later times that were not observed by our study.

50% FAM-labeled Amylin in pH 8 Polymer Run #1

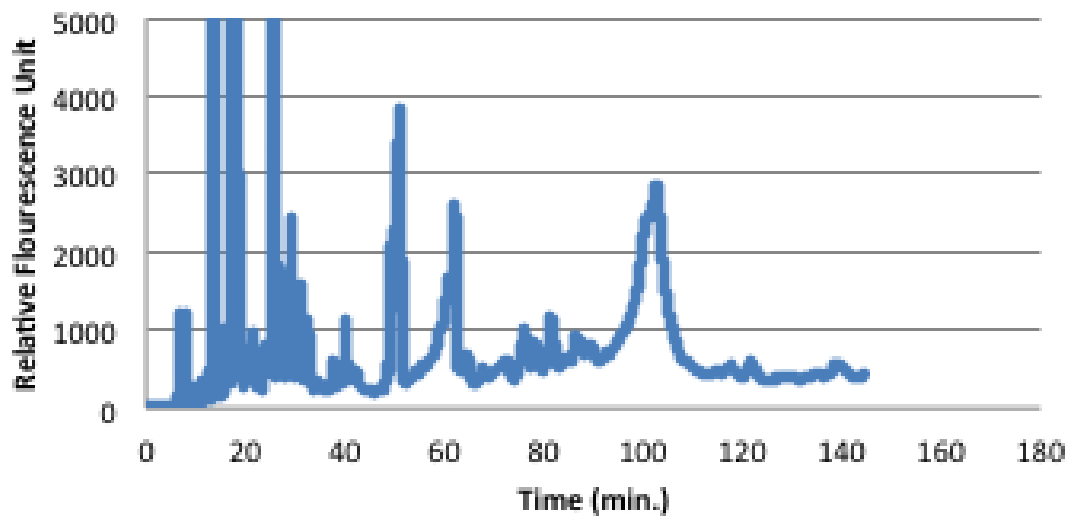
Run conducted using a ratio of 50% FAM labeled Amylin to 50% unlabeled Amylin.

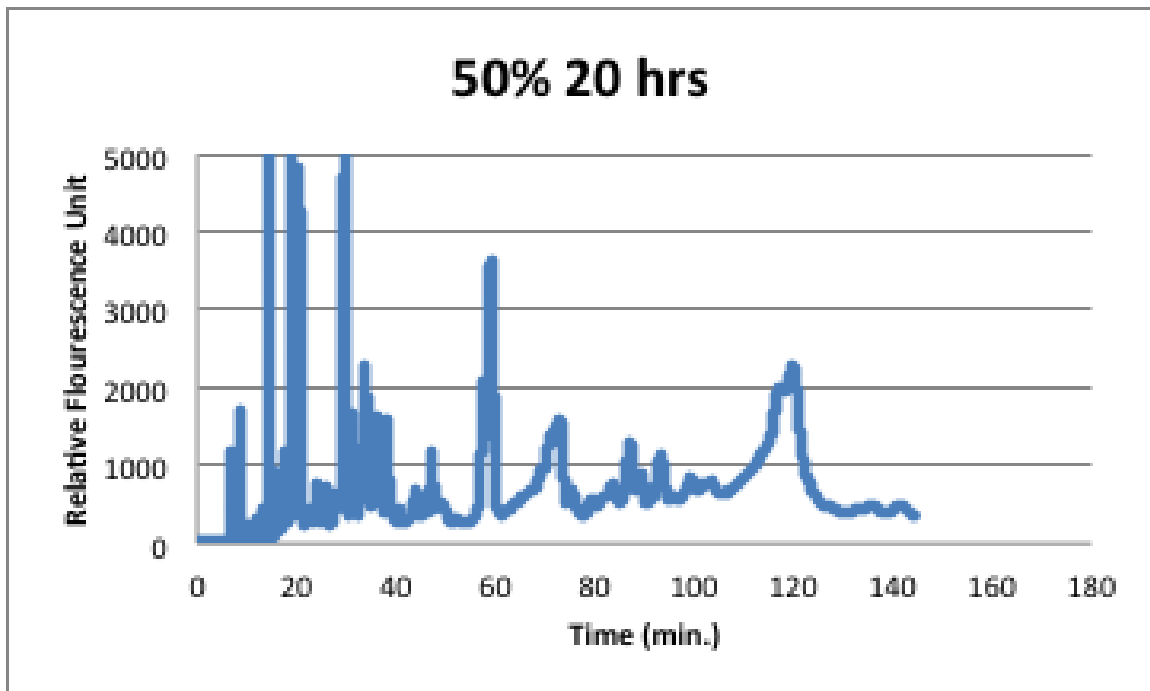
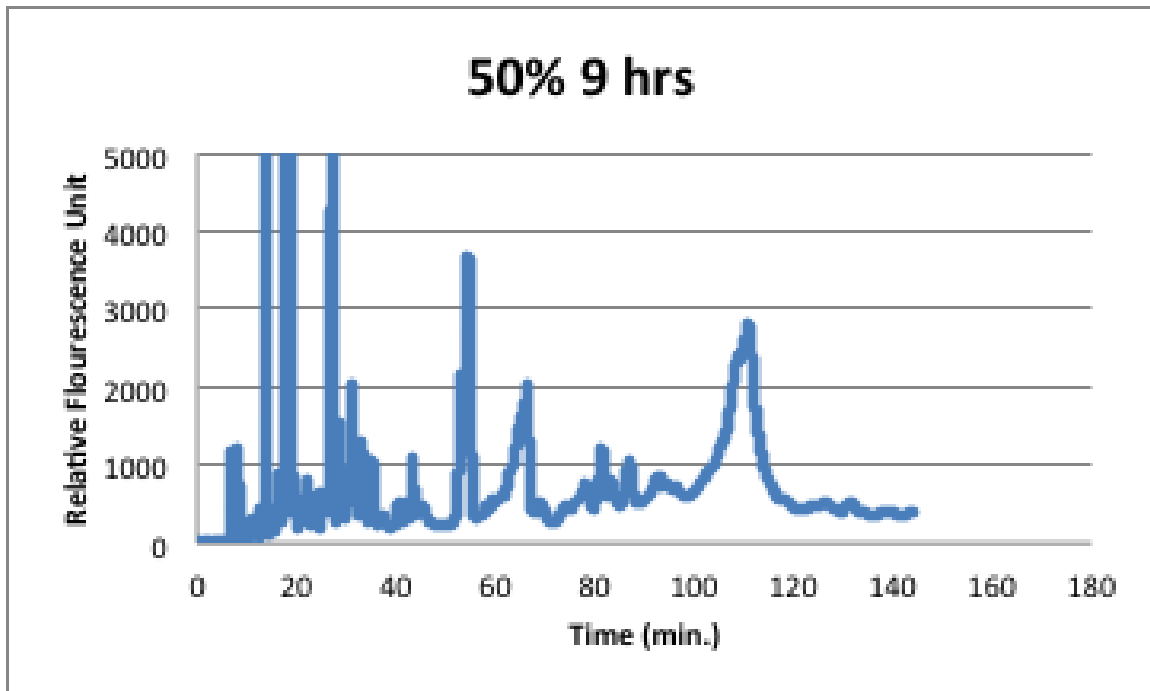


50% 3 hrs



50% 6 hrs

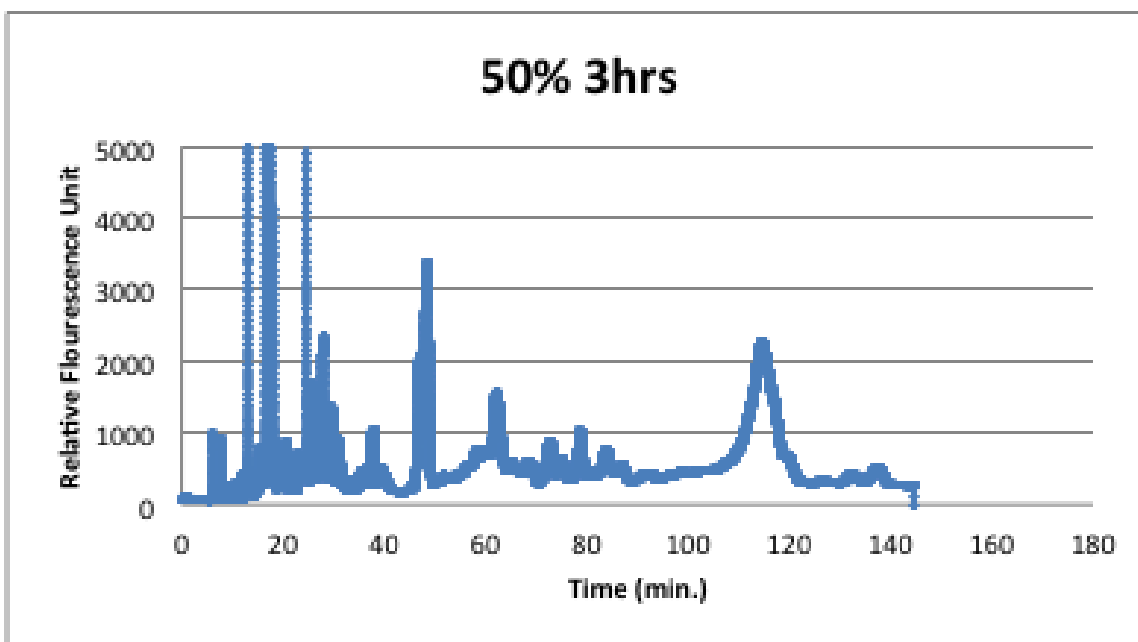
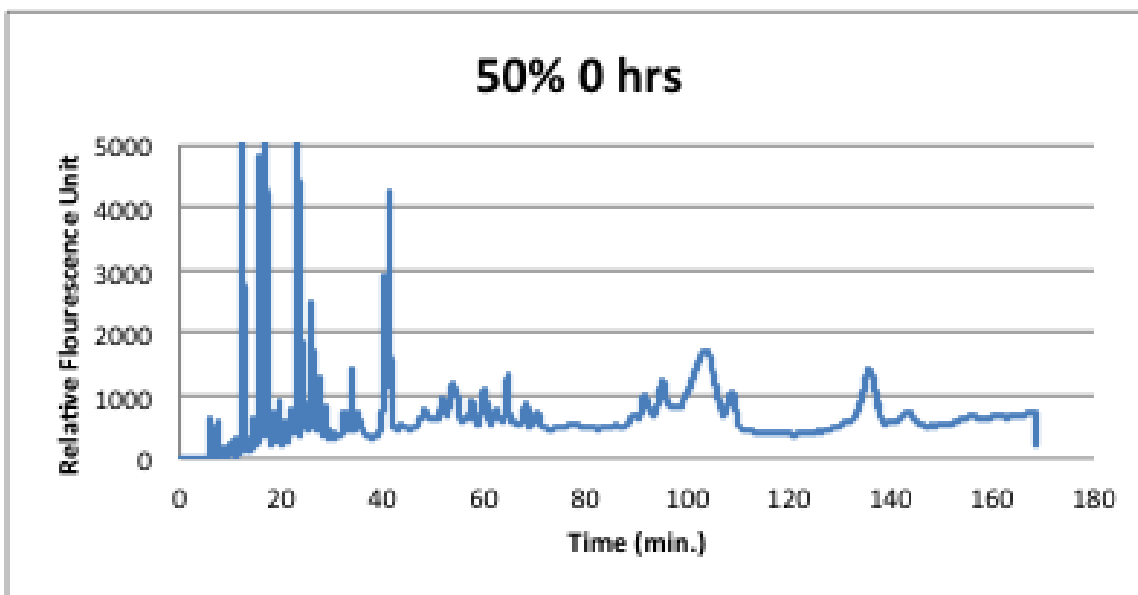




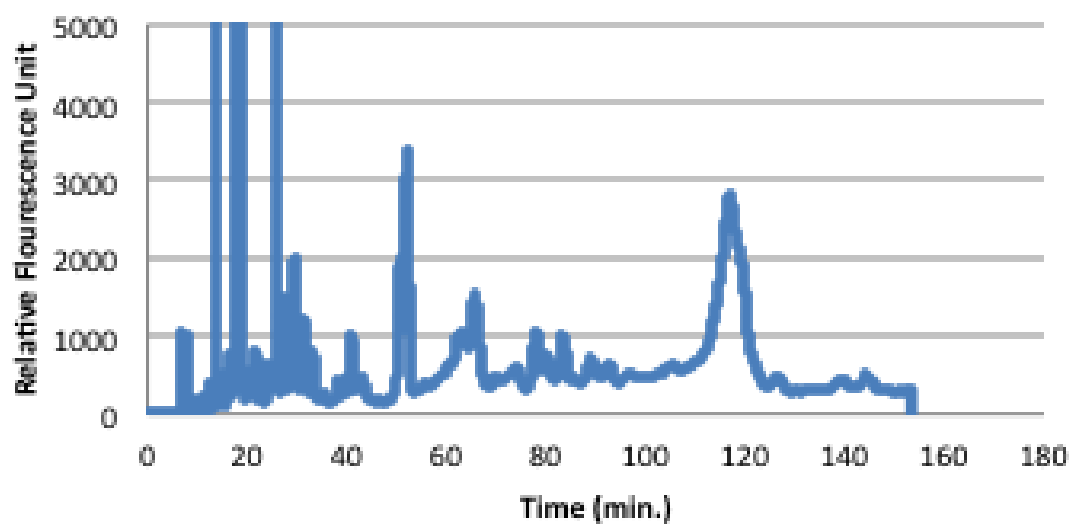
This 50% study shows three distinct peaks (40, 50 and 80 minutes in the 0 hr run). These three peaks each increase their elution time as the aggregation time increases from 0-24 hrs. An initial increase and then decrease in peaks area is observed as the aggregation time increases. Both of these observations suggest that larger species are forming as aggregation time increases.

50% FAM-labeled Amylin in pH 8 Polymer Run #2

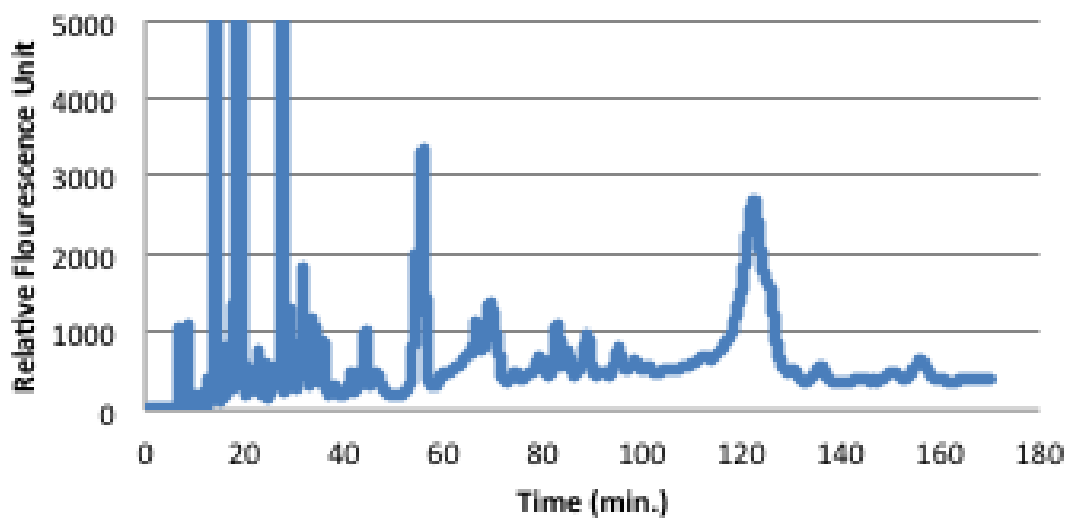
Run conducted using a ratio of 50% FAM labeled Amylin to 50% unlabeled Amylin

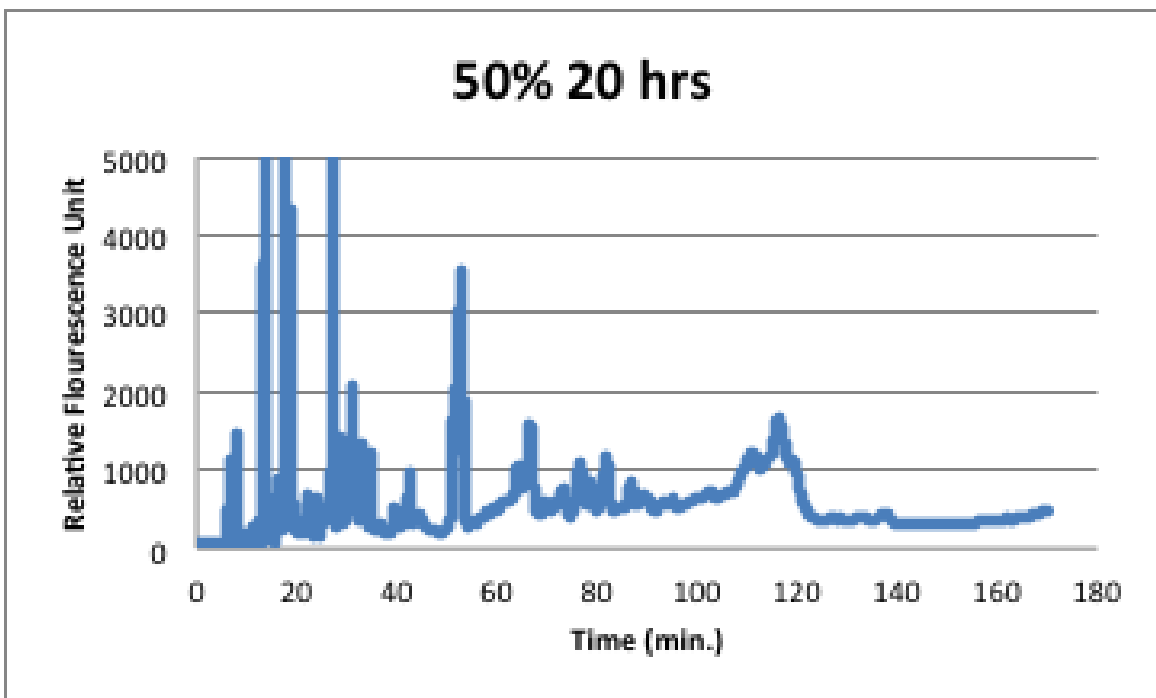


50% 6 hrs



50% 9 hrs



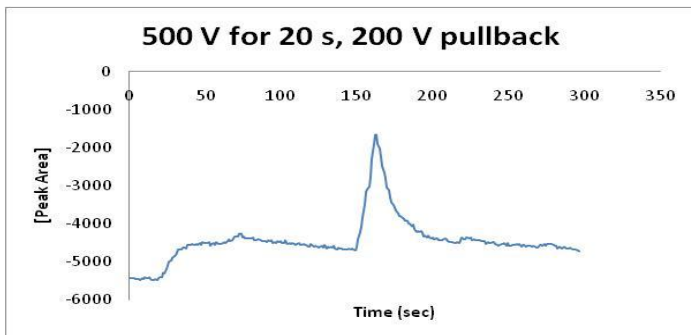


This 50% study also shows three distinct peaks (50, 60 and 120 minutes for the 20 hrs run). As with the other 50% study with pH 8 polymer, these three peaks each increase their elution time as the aggregation time increases from 0-24 hrs. An initial increase and then decrease in peaks area is observed as the aggregation time increases. Both of these observations suggest that larger species are forming as aggregation time increases.

Microchip Results

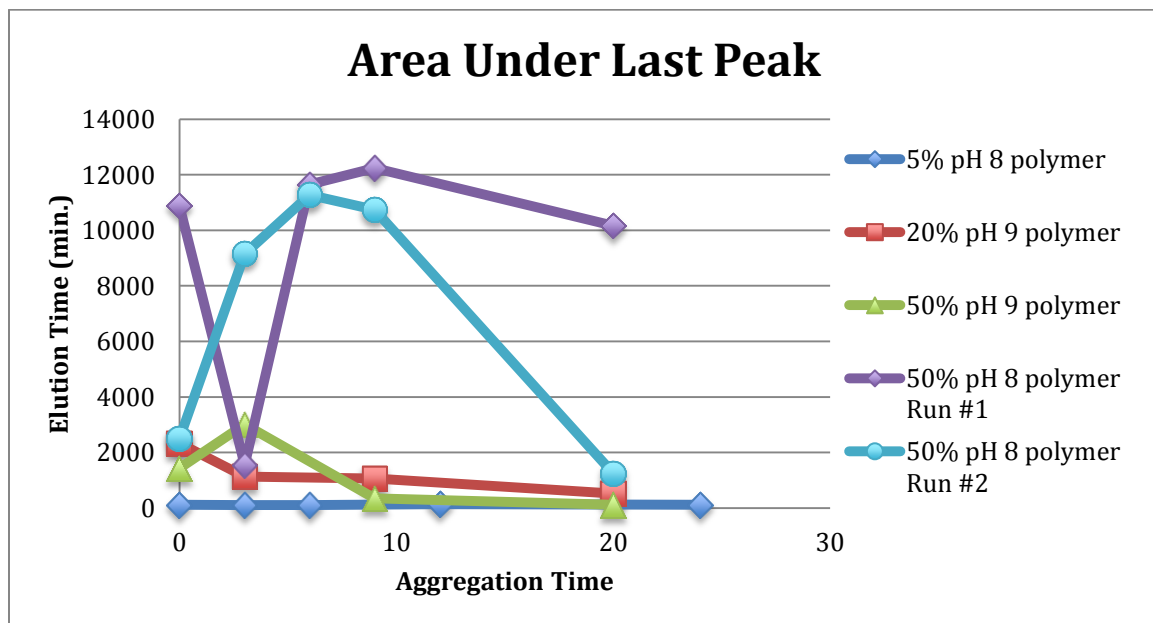
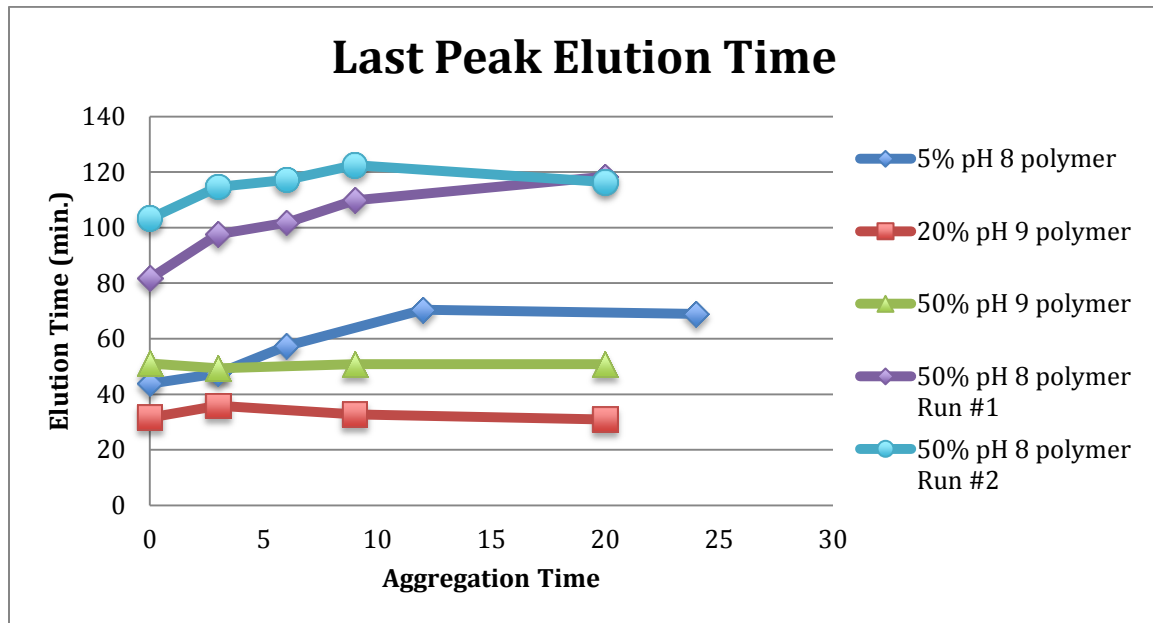
The microchip system was a home-built instrument that was only completed in the last months of the semester. Experiments were attempted at three different settings as stated in materials and methods. With all three runs no peaks were observed. Settings were changed with the two subsequent runs to attempt to get a greater gradient in the chip. Positive results were still not observed.

The following graph shows an example positive result for the microchip system. This was obtained by conducted a run with 100% FAM-AB(1-42).



Peak Analysis Studies

For the analysis of the runs, we wanted to look at how the protein grew over time. Two pieces of data can yield information about the growth. The mean elution time of the last peak at each time point can be calculated. This information shows whether or not larger species are in the solution as the aggregation time increases. The area under that last curve can also be measured. The last peak of each run was determined and a mean elution time and area under the curve were determined using data analysis software.



The last peak elution time graph shows an increase in size (elution time) of the last peak for all three studies that were conducted in the mixed pH condition (sample at pH 9, while polymer at pH 8). The two studies conducted in both pH 9 sample and polymer conditions did not show growth of the last peak. This analysis agrees with the visual results observed and discussed from the individual run graphs.

The result for area under the last peak showed similar findings. The area under the curve generally increased and then decreased for both runs conducted with 50% FAM-Amylin and pH 8 polymer. This also agrees with the visual studies from the individual run graphs. The purple curve point at 3 hrs that seems to show a significant drop is most likely an error. The increase in peak area seen in times up to ~9 hrs most likely indicated the growth of that size aggregate over time. The decrease in peak area at times >9 hrs is most likely due to the formation of an even larger species that did not elute before the run was ended. Again the pH 9 conditions did not show much change in peak area during the course of aggregation.

Based on these results, the pH 8-9 condition seems to have a favorable result for the protein aggregation. At this pH, the almost neutral Amylin will have very little driving force to move under the applied electric field. Two other factors could make the protein elute through the array. The FAM label does have a negative charge and could be pulling the Amylin through the array. The migration of the Amylin could also be due to the bulk electro-osmotic flow produced by the interaction of ions with the charged silanol groups on the capillary surface. The favorable aggregation of the Amylin at these conditions could be explained by its closeness to the protein isoelectric point (pI). Near the pI, Amylin has a neutral or very weakly negative charge. With little to no charge, the electrostatic effects between each individual protein becomes negligible and therefore the Amylin monomers will have a greater chance of coming together to form aggregates. Similar effects of low ionic strengths causing increased aggregation were observed in with other proteins. [14]

Conclusion

Aggregate growth was observed for Amylin protein. This shows that capillary electrophoresis is a potential method for studying the growth of Amylin oligomers. The technique can be used in the future to study the effects of inhibitors on Amylin growth and do other size characterization studies. Positive microchip results were not able to be obtained. There is still potential to optimize the conditions to obtain positive results. We did not have a large amount of time to try to optimize our runs on the system. It was only available for use near the end of this research.

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